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TITLE: Exploiting Tumor-Activated Testes Proteins To Enhance Efficacy of First-Line Chemotherapeutics in NSCLC

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**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

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**14. ABSTRACT**

Cancer Testis Antigens (CTAs) are a group of proteins whose expression is restricted to reproductive tissues (testis and ovaries), but frequently activated in Non Small Cell Lung Cancer (NSCLC). A number of these CTAs are essential for meiosis during spermatogenesis. Their deletion in mice leads to infertility due to an inability to repair DNA Double Strand breaks (DSB) during homologous recombination in meiosis. DSBs frequently occur in the tumorigenic environment due to environmental insults such as hypoxia and reactive oxygen species. We hypothesize that CTAs promote repair of these DSB in NSCLC and are essential for tumor cell survival. To evaluate this hypothesis, we are investigating the biochemical, cell biological and in vivo activity of CTAs in NSCLC. We have identified CTAs that are essential for DNA DB in NSCLC and which are also essential to survival of these cells in vitro. We find that the expression of meiotic CTAs appears to be a marker for the inactivation of metabolic pathways that lead to the generation of DNA-damage species. These findings are under testing in vivo. The physical interaction network of CTAs and identification of additional CTAs that may mediate sensitivity to DNA-damage inducing drugs is under investigation.

**15. SUBJECT TERMS**

Cancer Testis Antigen (CTA), Fanconia-Anemia (FA), DNA Damage, Genomic Instability, DNA Double Strand Break (DSB)

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
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1. **INTRODUCTION:** This proposal is focused on elucidating the function of Cancer-Testes Antigens (CTAs) in Non-Small Cell Lung Cancer. CTAs are genes whose expression is restricted to the testes but frequently reactivated in many tumor types. The purpose of this proposal is to elucidate the contribution of CTAs to the maintenance of genomic integrity at the molecular level and to examine whether CTAs modulate sensitivity to chemotherapy. To do this, a cohort of meiotic CTAs will be examined for their functional contribution to the activation of DNA damage repair pathways. These CTAs, TEX15, HORMAD1 and SYCP1 were implicated as important to NSCLC in preliminary studies. In addition, physical interactions between CTAs and components of the DNA repair machinery will be assessed. In vitro and in vivo studies will be used to assess the contribution of CTAs to tumor cell viability and chemosensitivity. A larger scale analysis of whether additional CTAs contribute to chemotherapeutic sensitivity will also be carried out.

2. **Keywords:**
    - Cancer Testis Antigen (CTA)
    - Fanconia-Anemia (FA)
    - DNA Damage
    - Genomic Instability
    - DNA Double Strand Break (DSB)

3. **Accomplishments**

- **What were the major goals and objectives of the project?**

**0-12 months:**

1) To analyze the FA pathway in CTA-depleted lung cancer cells (Vaziri Lab)
2) Complete Lentiviral-mediated CTA overexpression in HBE cells and analyze the FA pathway. (Vaziri Lab)
3) Complete dose curve and transfection optimization for a panel of NSCLC cell lines. (Whitehurst Lab)
4) Perform sensitivity assays for TEX15, SYCP1 and HORMAD1. (Whitehurst Lab)
5) Obtain IACUC approval for in vivo studies. (Whitehurst Lab)
6) Develop shRNA stable cell lines. (Whitehurst Lab)
7) Initiate in vivo studies. (Whitehurst Lab)

**12-24 months:**

8) Analyze ectopically-expressed epitope-tagged CTA in lung cancer cell lines. (Vaziri Lab)
9) Immunopurification and proteomic analysis of CTA complexes in cancer cells. (Vaziri Lab)
10) Screen for CTAs contributing to DNA damage. (Whitehurst Lab)
11) Continue in vivo studies. (Whitehurst Lab)

- **What was accomplished under these goals?** (Since this is a CO-PI grant, the descriptions are split into Aim 1 (Vaziri) and Aim 2 (Whitehurst) for the accomplishment section.

**AIM 1:** For each major goal proposed in SA1, major activities, specific objectives, and significant results/key outcomes are as follows:

**Task:** Analysis of ectopically-expressed epitope-tagged CTA in lung cancer cells (1.2).
**Major activities:** To express wild-type and mutant CTA in cultured cells and study the dynamic regulation of those CTA in relation to other elements of the DNA damage response.

**Specific objectives:** To define the proximal DSB-induced events that regulate CTA function following genotoxic injury, and to validate viral expression vectors to be used for proteomic studies.

**Significant Results:**

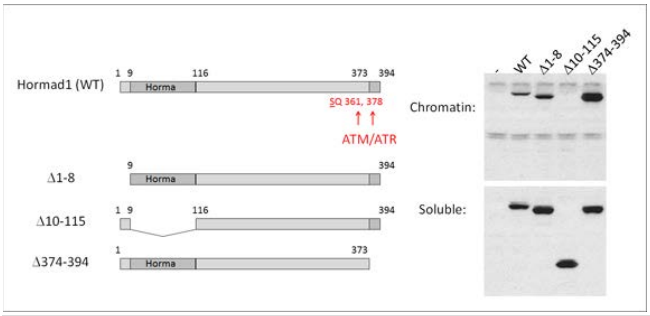


**Fig. 1** Expression and subcellular distribution of ectopically-expressed HORMAD1 mutants in lung

*Structure/function analysis of HORMAD1* - We generated a panel of HORMAD1 mutants harboring deletions and point mutations in various domains. WT and mutant forms of HORMAD1 were introduced into cultured lung carcinoma cells using adenovirus vectors. Several HORMAD1 mutants did not express (most probably due to protein misfolding and/or instability). Mutant proteins that expressed at levels similar to WT HORMAD1 (some of which are described in **Fig. 1**) are currently being studied for responsiveness to DNA DSB and ability to support Homologous Recombination and radioresistance in cells. It is anticipated that these studies will identify key functional domains of HORMAD1 and identify mechanisms of responsiveness to DSB as well as the molecular mechanism by which HORMAD1 interfaces with the HR machinery.

**Task: Immuno-purification and proteomic analysis of CTA complexes in lung cancer cells (1.2)**
**Major Activities:** We performed unbiased proteomic screens for HORMAD1-assocaited proteins. For those experiments lysates from HA-epitope-tagged HORMAD1-expressing cells (or control cultures not expressing ectopic HORMAD1) were immunoprecipitated with anti-HA antibodies and the resulting immune complexes were analyzed by mass spectrometry.

**Specific Goals:** To define activators and effectors of HORMAD1 in DSB repair

**Significant Results:** Initial mass spectrometry experiments revealed enrichment of several structural nuclear proteins (spectrin and actinin) in HA-HORMAD1 immunoprecipitates relative to control pulldowns. However, in independent experiments we were unable to validate specific association of any of these potential binding partners with HORMAD1.

**Changes in Approach or Methods:** Because our standard experimental approach for defining PIN of DNA repair proteins has not identified specific HORMAD1-binding partners we are repeating IP/mass spec experiments using lower-level HORMAD1 expression and more stringent IP conditions to eliminate non-specific binders. As an additional strategy for eliminating false positives, we will perform IP/mass spec experiments using some of the inactive mutants identified in our structure/function analyses. The rationale is that for our validation experiments we will focus on interactors that bind to the WT HORMAD1 but not mutant forms of HORMAD1 that are inactive in DSB signaling and HR.

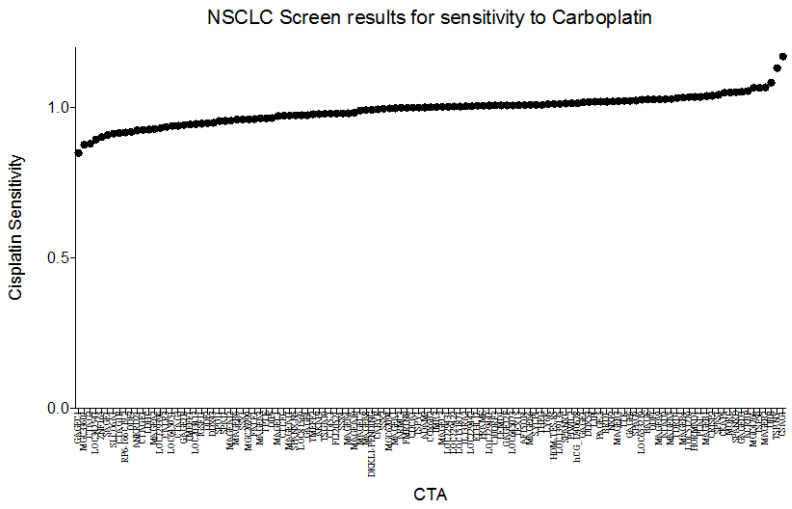


**Fig. 2** Results of siRNA screen to identify sensitivity to Carboplating in NSCLC.

**AIM 2:** The Whitehurst lab activities have been focused on Aim 2, in which we will assess dependency for survival of NSCLC on CTAs for survival or chemosensitivity of NSCLC in vitro and in vivo.

For each major goal proposed in SA2, major activities, specific objectives, and significant results/key outcomes are as follows:

**Task: Screen for CTAs contributing to DNA damage. ( Aim 2.3)**
**Major activities:** Screening of CTAs to identify those that may sensitize to DNA damage agents in NSCLC.
**Specific Objectives.** To identify CTAs that are essential for mitigating the response to first-line chemotherapeutic agents.

**Significant results:** Using an siRNA based approach we screened ~105 CTAs but did not observe any significant alterations in sensitivity to Cisplatin (Figure 2). This result could mean that no single CTA is capable of significantly sensitizing cells to carboplatin or that our assay conditions were not sensitive enough to detect changes. **Changes in approach or methods:** Based on this finding, we reevaluate our analysis on HORMAD1, which we could during the first year of work could support viability in certain settings as well as mediate radioresistance in at least one NSCLC setting. Furthermore, we found that cells that express HORMAD1 are resistant to Piericidin A, which is an electron transport chain disrupter, that leads to leakage of reactive oxygen species and downstream DNA damage. We most recently found that HORMAD1 expression is present in nearly 40 % of NSCLC and these patients have a significantly poorer survival and also have an elevated mutation burden, suggesting chronic DNA damage (Figure 3). We observed **no other CTA** that exhibited this robust pattern of expression, correlation with survival or mutation burden or chemical sensitivity. Taken together, the siRNA results along with those for HORMAD1, suggest that HORMAD1 is the key CTA that is mediating DNA damage response in a subset of NSCLC. This can be best observed by radiosensitization assay, which is not amenable to high-throughout screening. Based on initial results that HORMAD1 is essential for radiosensitivity and in particular, for Homologous Recombination, we are currently screening for radiosensitivity in a panel of HORMAD1 positive cell lines to evaluate whether these cell lines exhibit comparatively elevated resistance to radiosensitivity and to cisplatin. **If this association holds, this would suggest the exciting possibility that HORMAD1 is a biomarker for resistance to DNA damage in patient populations and could suggest alternative modes of treatment.**

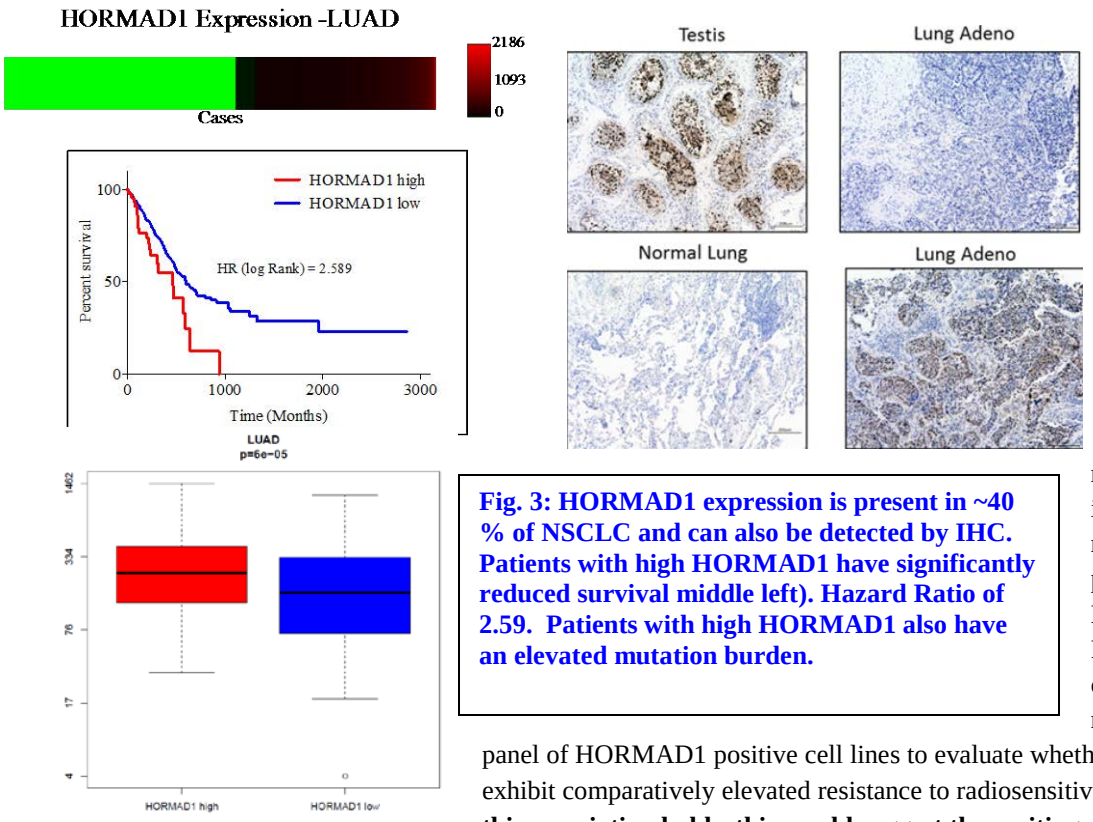


**Fig. 3: HORMAD1 expression is present in ~40 % of NSCLC and can also be detected by IHC. Patients with high HORMAD1 have significantly reduced survival middle left). Hazard Ratio of 2.59. Patients with high HORMAD1 also have an elevated mutation burden.**

**Task: Continue in vivo studies to examine the consequences of CTAs on growth in vivo. (Whitehurst Lab) (Aim 2.2)**
**Major Activities:** Implant NSCLC tumors depleted of HORMAD1 and examine the consequences on growth in vivo and sensitivity to DNA damage agents.
**Specific Objective:** To determine whether CTAs can influence the growth of tumors in vivo.
**Significant Results:** We used a variety of gain and loss of function approaches to first determine if HORMAD1 is essential for tumorigenesis in vivo. Here, we found that either gain of HORMAD1 in a null background (NSCLC that does not express HORMAD1) or loss of HORMAD1 does not impact tumorigenesis. This finding suggests that HORMAD1 is not required for the transformation event (Figure 4). Thus, we are now evaluating the consequences of HORMAD1 loss or gain on sensitivity to chemotherapeutic agents in vivo. Based on our findings that Homologous Recombination is impacted by HORMAD1 loss in vitro, we are using Cisplatin for these studies. We have developed doses (30-60 mg/kg) that appear to be appropriate for in vivo studies based on published data and our own in vivo analysis. In the next year, we will continue with these studies to determine if HORMAD1 is required for in vivo sensitivity to cisplatin and also if cells that express HORMAD1 are more resistant to cisplatin when grown in vivo. These studies will provide important information on the regulation of HORMAD1 in vivo. **Changes to approach or Methods:** No deviations from the original proposal.

**Task: Screen for CTAs contributing to DNA damage. (Whitehurst Lab)**

**Changes in Approach or Methods:** As evident from the description above, there has been no significant change in approach or methods from the agency approved application or plan.

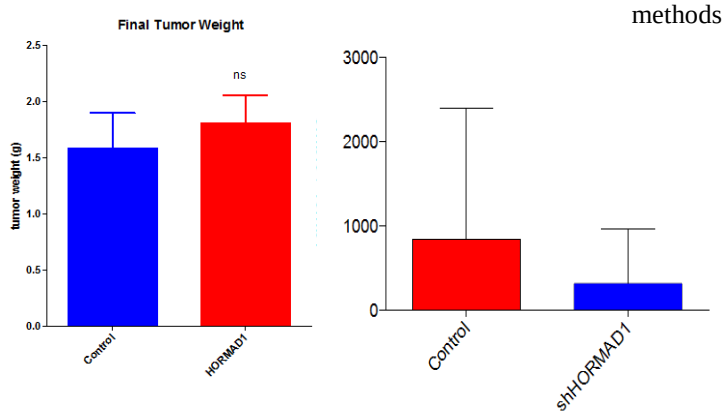


**Fig. 4: Consequences of HORMAD1 expression on growth in vivo. Left panel: Overexpression of HORMAD1 in a null background did no enhance growth significantly. Right: Loss of HORMAD1 (shHORMAD1) did not significantly reduce tumor growth in vivo).**

**Adherence to original timetable:** As expected, we have largely completed the analysis proposed in the two years. However, as a few of the experiments did not proceed as expected, we have had to alter or delay some approaches. In the No cost extension period we will further performs screens to understand HORMAd1 resistance and complete the vivo studies to analyze chemoresistance of HORMAD1 tumors in vivo.

**What opportunities for training and professional development has the project provided?**

A post-doctoral fellow at UNC (Yanzhe Gao) performed the studies in support of SA1. His training activities included one-on-one work with Dr. Vaziri and acquisition of new imaging skills in deconvolution fluorescence microscopy (1.2). At UTSW, Jennifer Macion has been performing studies in support of Aim 2. This allowed her to gain new skills with respect to cell biology and screening analysis.

**How were the results disseminated to communities of interest?**

Nothing to Report

**What do you plan to do during the next reporting period to accomplish the goals?**

As described above, we will continue to evaluate the HORMAD1 positive cohort and screen this cohort for radio and chemo-sensitivity to DNA damage agents and evaluate the requirement for HORMAD1. We will also determine if additional CTAs are essential for the HORMAD1 mediated process. We will further evaluate these findings in vivo during the no cost extension period.

**4. Impact**

Nothing to report.

**5. CHANGES/PROBLEMS**

Nothing to report.

**6. PRODUCTS:**

Nothing to report.

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort).

| | |
|---|---|
| **Name:** | **Angelique Whitehurst** |
| Project Role: | PI |
| Researcher Identifier (e.g. ORCID ID): | N/A |
| Nearest person month worked: | 1 |
| Contribution to Project: | Wrote SA1 of original proposal and supervised experiments in support of SA1 |
| Funding Support: | This award, federal, private and institutional funds. |

| | |
|---|---|
| **Name:** | **Jennifer Macion** |
| Project Role: | Research Technician |
| Researcher Identifier (e.g. ORCID ID): | N/A |
| Nearest person month worked: | 11 |
| Contribution to Project: | Jennifer Macion worked on the experiments in Aim 2. |
| Funding Support: | This award, federal and private foundation funds. |

| | |
|---|---|
| **Name:** | **Cyrus Vaziri** |
| Project Role: | PI (at partner site, UNC) |
| Researcher Identifier (e.g. ORCID ID): | N/A |
| Nearest person month worked: | 1 |
| Contribution to Project: | Wrote SA1 of original proposal and supervised experiments in support of SA1 |
| Funding Support: | NIH awards and partial support from this award |

| | |
|---|---|
| **Name:** | **Yanzhe Gao** |
| Project Role: | Post-doc (at partner site, UNC) |
| Researcher Identifier (e.g. ORCID ID): | N/A |
| Nearest person month worked: | 11 |
| Contribution to Project: | Dr. Gao performed all the experiments described under 'work accomplished' |
| Funding Support: | This award and Start-up funds to Vaziri from UNC |

**Has there been a change in the active other support of the PD/PIs since the last reporting period?**
Nothing to Report

**What other organizations were involved as partners?**
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina

**Partner's Contribution to the project:** Collaboration

**8. SPECIAL REPORTING REQUIREMENTS:** None

**9. APPENDICES:** (Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.) None